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Ataxin-10 interacts with O-GlcNAc transferase OGT in pancreatic β cells

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Abstract

Several nuclear and cytoplasmic proteins in metazoans are modified by O-linked *N*-acetylglucosamine (O-GlcNAc). This modification is dynamic and reversible similar to phosphorylation and is catalyzed by the O-linked GlcNAc transferase (OGT). Hyperglycemia has been shown to increase O-GlcNAc levels in pancreatic β cells, which appears to interfere with β-cell function. To obtain a better understanding of the role of O-linked GlcNAc modification in β cells, we have isolated OGT interacting proteins from a cDNA library made from the mouse insulinoma MIN6 cell line. We describe here the identification of Ataxin-10, encoded by the SCA10 (spinocerebellar ataxia type 10) gene as an OGT interacting protein. Mutations in the SCA10 gene cause progressive cerebellar ataxias and seizures. We demonstrate that SCA10 interacts with OGT in vivo and is modified by O-linked glycosylation in MIN6 cells, suggesting a novel role for the Ataxin-10 protein in pancreatic β cells.

Keywords: MIN6; β cells; SCA10; OGT; Glucose; Diabetes; Ataxia; O-GlcNAc; Ataxin-10

O-linked GlcNAc modification has been observed on numerous cytosolic and nuclear proteins [1–3]. This dynamic and reversible modification can alter the function of proteins by modulating enzyme activity, subcellular localization, protein–protein interaction, DNA binding, and proteolytic processing [1–3]. O-linked GlcNAc modification consists of the addition of a single *N*-acetylglucosamine sugar to serine or threonine residues on nuclear and cytosolic proteins by the enzyme O-linked GlcNAc transferase (OGT). OGT appears to have a wide range of tissue distribution, although the levels of expression and activity are not correlative. It contains two putative domains, a tetratricopeptide repeat (TPR) at the amino-terminus, which is important for the interaction of OGT with its substrate proteins, and a catalytic C-terminal domain [4–7]. A recent study reported the existence of a mitochondrial form of OGT, which appears to be a splice variant [8]. However, the function and specificity of this splice variant remains unknown.

Previous studies indicate that the O-GlcNAc levels on key cellular proteins are modulated by changes in the extracellular glucose concentration [9]. High concentrations of glucose have been shown to increase O-GlcNAc levels in pancreatic β cells [10,11]. These findings led to the proposal that the O-GlcNAc transferase OGT is a nutritional sensor and that the cell globally modulates its behavior by regulating O-GlcNAc levels in response to its nutritional state [9–12]. Since chronic hyperglycemia has been shown to increase the levels of O-linked GlcNAc modified proteins, it has been suggested that abnormal modification of proteins may contribute to the pathogenesis of diabetes. To gain a better understanding of the role of O-GlcNAc modification in pancreatic β cells, we have performed a yeast Cytotrap screen to identify OGT interacting proteins. Using a cDNA library prepared from the mouse insulinoma 6 (MIN6) cell line, we have isolated Ataxin-10 (spinocerebellar ataxia type 10 (SCA10)) as one of the OGT interacting proteins.

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Mutations in the SCA10 gene cause "autosomal dominant cerebellar ataxias" (ADCAs) [13,14] and result from an expansion of a pentanucleotide repeat (ATTCT) in intron 9 of the SCA10 gene [15]. Ataxin-10 is a helical armadillo-like protein that is predicted to have a similar three-dimensional structure as Huntington. The armadillo repeat motif usually confers protein–protein interactions with diverse cellular binding partners and elicits functions in many different biological contexts [16]. The observation that Ataxin-10 interacts with OGT and is O-GlcNAc modified only at high concentrations of glucose suggests a novel role for this protein in glucose regulation of β cell function.

Experimental procedures

Isolation of OGT-interacting proteins using the yeast Cytotrap screen. SCA10 was isolated as an OGT-interacting protein using the CytoTrap system according to the manufacturer's protocols (Stratagene) [17,18]. For this purpose the mitochondrial version of human OGT [4,8] was subcloned into the pSos vector (Stratagene) in-frame with the human Sos protein and used as bait. To carry out the screening, we have constructed a cDNA library from the mouse insulinoma (MIN6) cell line in the pMyr vector in frame with the myristoylation signal (Stratagene) [17–19]. The screen for OGT interacting proteins was performed in the temperature sensitive yeast strain cdc25H according manufacturer's protocols (Stratagene). After screening of about 1×10^6 colonies, the SCA10 gene was isolated three independent times as an OGT interacting protein.

Cell culture. Mouse insulinoma 6 (MIN6) cells of passage 19 through 30 were cultured in Dulbecco's modified Eagle's media (DMEM) containing 5 mM glucose, 10% (vol/vol) fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM glutamine, and 100 μM β-mercaptoethanol [19]. For the glucose regulation experiments, MIN6 cells were incubated with 1 or 25 mM glucose containing media for 14–16 h.

Co-immunoprecipitation assays. For co-immunoprecipitation assays, whole cell extracts (about 0.5–1 mg) from MIN6 cells incubated with 1 or 25 mM glucose were prepared in lysis buffer (10 mM Tris–Cl, pH 8.0, 140 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P-40, 20% glycerol, 1 mM PMSF, and protease inhibitors) and diluted with four volumes of dilution buffer (50 mM Tris pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors). Immunoprecipitation with specific antibodies was carried out overnight at 4 °C followed by incubation with protein A–Sepharose 4 Fast Flow (Amersham Pharmacia) for 1–2 h. The pellets were washed six times in 1 ml wash buffer (50 mM Tris–Cl, pH 7.5, 10% glycerol, 100 mM NaC1, 0.1% NP-40, and 1 mM EDTA) and resuspended in 2× SDS sample buffer. Non-immunized rabbit IgG (Sigma) was used as a negative control for non-specific binding. The proteins were resolved on 10% SDS–PAGE, transferred onto nitrocellulose membrane, and immunoblotted with specific antibodies. Proteins were visualized using the ECL chemiluminescent detection system (Amersham Biosciences).

Generation of OGT and Ataxin-10 antibodies. OGT and Ataxin-10 peptide antibodies were produced in rabbits (Genemed Synthesis Inc.). The OGT peptide used as an antigen included the last 12 carboxyl-terminal amino acids of human OGT. Ataxin-10 peptide antibodies were produced using two different peptides, peptide 1 covering the amino acids 329–343 and peptide 2, the amino acids 370–383 of the rat Ataxin-10 protein.

In vitro interaction assays. For pull-down experiments, 1 μg of recombinant Gst-OGT was incubated with glutathione–agarose beads (Amersham Biosciences) for 1 h. After washing the beads four times with binding buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaC1, 0.1% NP-40, 2 mM EDTA, and 10 mM $MgCl_2$), 1 μg of the His-tagged Ataxin-10 protein was added and incubated with rotation at 4 °C for 1 h. The beads were then washed four times with binding buffer and the proteins were subsequently eluted from the beads by boiling them for 10 min in 1× SDS loading buffer. The reverse pull-down experiments were performed using the same procedure, but by incubating 1 μg recombinant His_6-Ataxin-10 with Ni–NTA agarose. For these experiments the binding buffer also included 20 mM imidazole.

Results

O-linked N-acetylglucosamine transferase interacts with Ataxin-10 in the yeast cytotrap screen

To identify O-linked *N*-acetylglucosamine transferase (OGT) interacting proteins from pancreatic β cells, we have used the Yeast Cytotrap screen, in which the interaction occurs in the cytoplasm. For this purpose, the mitochondrial version of human OGT was subcloned into the bait plasmid pSos in-frame with the human Sos protein and the screen was carried out with a cDNA library made from the mouse insulinoma MIN6 cell line according to manufacturer's protocols (Stratagene). After screening of about 1×10^6 yeast colonies with hSos-OGT as bait, SCA10 was isolated as an interacting protein three independent times.

To confirm the interaction between SCA10 and OGT, we have transformed the temperature sensitive cdc25H yeast strain with Myr-SCA10 and Sos-OGT, Myr-SCA10 and Sos, and Myr and Sos-OGT constructs. After growing the transformants on YNB glucose without leucine and uracil at the permissive temperature of 30 °C, they were replica plated onto YNB galactose without leucine and uracil, and incubated at 37 °C (restrictive temperature) (Fig. 1). At 37 °C only cdc25H transformed with Sos-OGT + Myr-SCA10 grew (Fig. 1), suggesting an interaction between OGT and SCA10.

Ataxin-10 interacts with OGT in MIN6 cells

Since Ataxin-10 was isolated as an OGT-interacting protein in the yeast Cytotrap screen, we tested whether Ataxin-10 co-immunoprecipitates with OGT in MIN6 cells. Extracts from MIN6 cells incubated with 1 or 25 mM glucose for 16 h were prepared and used in

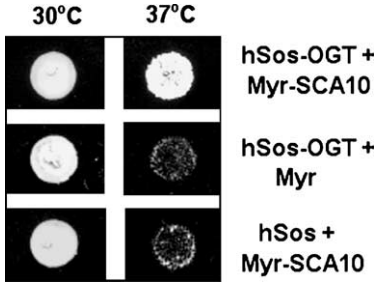

Fig. 1. OGT interacts with SCA10 in the yeast Cytotrap screen. The cdc25H strain was transformed with hSos-OGT plus Myr-SCA10 or with hSos-OGT plus Myr or hSos plus Myr-SCA10 and the obtained transformants were replica plated on YNB-galactose media without leucine and uracil, and incubated at 30 °C (permissive temperature) or 37 °C (restrictive temperature).

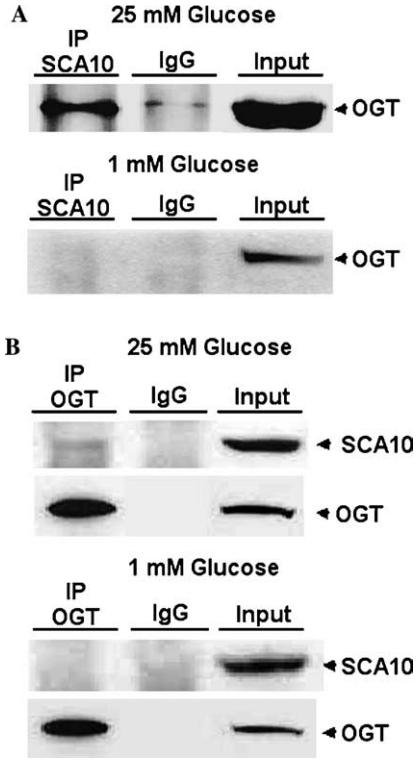

Fig. 2. Ataxin-10 interacts with OGT in MIN6 cells incubated only with high glucose. Protein extracts from MIN6 cells grown on 1 or 25 mM glucose overnight were immunoprecipitated with Ataxin-10 (IP-SCA10, A) or with OGT antibodies (IP-OGT, B) and immunoblotted for OGT or Ataxin-10 protein. As a negative control, the immunoprecipitation assays were also carried out with rabbit IgG.

co-immunoprecipitation experiments with Ataxin-10 antibodies. Western blot analysis of Ataxin-10 co-immunoprecipitated proteins with OGT antibodies indicated that Ataxin-10 is associated with OGT in MIN6 cells on high glucose but not on low glucose (Fig. 2A). Co-immunoprecipitation experiments carried out with OGT antibodies also confirmed the interaction of OGT with Ataxin-10 on high glucose (Fig. 2B). Consistent with the idea that OGT and Ataxin-10 interact with each other, the expression pattern for both proteins is very similar with highest levels present in the testis, cortex, and cerebellum (data not shown).

OGT interacts with Ataxin-10 in vitro

In order to confirm that the interaction between OGT and Ataxin-10 was direct, recombinant His_6-tagged Ataxin-10 (His_6-SCA-10) and Gst-tagged OGT (Gst-OGT) were produced and purified from bacteria. Approximately 1 μg of His_6-SCA-10 was incubated with equal concentrations of Gst-OGT as described under Experimental procedures. Pull-down assays were performed by adding either Ni–NTA or glutathione beads to the reaction mixture. Ataxin-10 was able to interact with Gst-OGT in vitro and there was no interaction between Ataxin-10 and Gst alone (Fig. 3). These data indicate that Ataxin-10 interacts with the OGT directly.

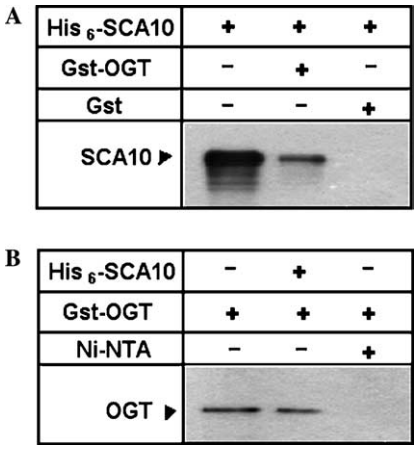

Fig. 3. OGT interacts with Ataxin-10 in vitro. The interaction of recombinant His_6-SCA10 with Gst-OGT was determined by incubating about 1 μg of purified protein for 1 h in the presence of GSH beads (A) or Ni–NTA agarose (B). After washing, the samples were separated on 10% SDS–PAGE and blotted for SCA10 (A) or OGT (B). Purified Gst or Ni–NTA agarose were used as negative controls.

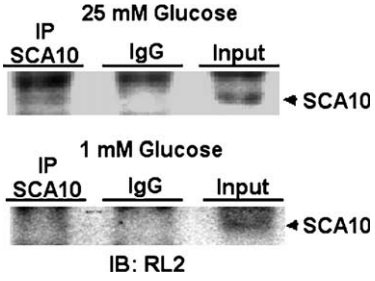

Fig. 4. Ataxin-10 is O-GlcNAc modified in MIN6 cells incubated with high glucose. MIN6 cell extracts incubated with 1 or 25 mM glucose were used for immunoprecipitation with Ataxin-10 antibodies. The immunoprecipitates were then blotted with RL2 antibodies that recognize O-GlcNAc residues on proteins.

Ataxin-10 is modified by O-linked GlcNAcylation only in response to high levels of glucose

To test whether Ataxin-10 is a substrate for OGT, we immunoprecipitated Ataxin-10 from low (1 mM) or high (25 mM) glucose incubated MIN6 cell extracts. The obtained immunoprecipitates were immunoblotted with the RL-2 antibody, that recognizes O-GlcNAc modification on proteins [20] and found that Ataxin-10 was modified by O-GlcNAc attachment only on high glucose (Fig. 4). Since Ataxin-10 interacts with OGT and it is modified by O-GlcNAcylation only on high glucose, we analyzed the Ataxin-10 levels in MIN6 cells grown on low (1 mM) or high (25 mM) glucose. The level of Ataxin-10 protein did not significantly change by treatment with different amounts of glucose (data not shown).

Discussion

Diabetes mellitus is caused by inappropriate glucose metabolism leading to impaired removal of glucose from

the circulation, which results in hyperglycemia. Recently, increased O-GlcNAc modification of proteins in response to hyperglycemia has been implicated in the etiology of type 2 diabetes. The O-GlcNAc transferase OGT has been shown to be ubiquitously expressed in almost all human and mouse tissues, and the number of O-GlcNAc-modified nuclear and cytoplasmic proteins identified has reached nearly 100, which includes transcription factors, cytoskeletal components, metabolic enzymes, and signaling components [1–3,21]. In the present study, we report the identification of Ataxin-10 as an OGT-interacting protein isolated from a library made from the mouse insulinoma cell line MIN6. The interaction of Ataxin-10 with OGT and its modification by O-GlcNAcylation in MIN6 cells occur only on high glucose. These data indicate that Ataxin-10 is a substrate for OGT in pancreatic β cells and is modified by OGT only in response to increased glucose levels.

Mutations in SCA10 gene are associated with autosomal dominant neurodegenerative disorders characterized by ataxia, seizures, and anticipation [15,22,23]. The mutations in the SCA10 gene are due to pentanucleotide (ATTCT) repeat expansions and the dynamic property of nucleotide-repeat expansion is thought to contribute to variable manifestation of this disease [15,24]. The function of the Ataxin-10 protein either in normal physiology or in disease states remains unknown. Recent data indicate that depletion of Ataxin-10 in neuronal PC12 cells leads to cell death, suggesting a role for Ataxin-10 in cell survival [25]. It is possible that Ataxin-10 is also important for cell survival in pancreatic β cells in response to changes in glucose levels.

There are no studies available linking SCA10 mutations to diabetes. However, patients suffering from the disease Friedrich's ataxia show primarily increased insulin secretion during the nondiabetic phase, which then turns into impaired insulin secretion accompanied by diabetes mellitus. More than 100 inherited syndromes of neurodegeneration have now been described [26], of which more than 20 are associated with diabetes mellitus. Huntington patients develop diabetes mellitus about seven times more often than matched healthy control individuals [27]. The reason for this concomitant disorder is unclear, although inappropriate insulin secretion is a potential reason [28]. Among ataxias, spinocerebellar ataxia 3 and 6, which are characterized by ataxia, spasticity, and aberrant ocular movements [27], were found to be associated with diabetes mellitus. Most of the above-mentioned neurodegenerative disorders are typically or frequently associated with diabetes mellitus or its antecessors, insulin resistance, and/or impaired glucose tolerance. Neurons and pancreatic β cells have high metabolic properties which make these cells extremely sensitive to genetic and environmental stresses, causing clinically evident damage, while other tissues remain functional. Hence, it is likely that typical neurodegenerative disorders and type 2 diabetes share common genetic and biochemical features. Consistent with this idea, we propose that Ataxin-10, which appears to be involved in cell survival in neurons [25] may also play an important role in survival of pancreatic β cells.

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